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# Interferon and Growth Factor Modulation of Nuclear Factors Binding to 5′ Upstream Elements of the 2-5A Synthetase Gene

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We assayed fragments of the 5′ flanking sequence of the human 2–5A synthetase gene for their ability to respond to interferon-α (IFN) and platelet-derived growth factor (PDGF). Transient transfection assays identified a 40-base pair fragment, which, regardless of orientation, could confer IFN-inducibility on the thymidine kinase promoter. This same fragment was active in monkey and mouse cells and in the latter was responsive to PDGF. The effect of PDGF could be inhibited by anti-interferon antibodies. Gel retardation assays, using the 40-base pair probe, detected the presence of IFN-modulated DNA-binding factors in nuclear extracts from monkey cells. In mouse cells both IFN and PDGF induced the binding of nuclear factors to a synthetic 2–5A synthetase response sequence. Thus, both IFN and growth factors directly or indirectly modulate the binding of nuclear factors to the same region of the 2–5A synthetase gene.

**Key words: platelet extract, PDGF, chloramphenicol acetyl transferase, gel retardation, transfection, induced expression**

Interferons (IFN) may play an important role as potent polypeptide antigrowth factors. Acting in an autocrine or paracrine manner and interacting with growth factors and other regulatory cytokines, they likely provide a tightly controlled mechanism for regulating cell growth. The interaction of IFNs with specific high-affinity receptors in the plasma membrane results in the induction of a number of genes. One of these genes, that encoding 2–5A synthetase, has been characterized in detail [1–4], and the transcriptional activation by IFN has been shown to parallel closely IFN-receptor interactions [5]. Interestingly, this gene is also induced by PDGF apparently as a primary response [6] (M. Garcia Blanco and C.D. Stiles, pers. commun.). This has led to the suggestion that the 2–5A synthetase pathway may be involved in regulating the mitogenic response to growth factors, perhaps in an autocrine loop [6]. This would occur through the synthesis of 2′-5′-linked oligomers of adenosine

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(2–5A), which activate a latent endoribonuclease that degrades mRNA [reviewed in 7]. The 2–5A is rapidly degraded, so the activation of the nuclease and inhibition of protein synthesis is transient. Thus, the 2–5A system could provide a possible mechanism for limiting the proliferative response of cells to mitogens.

There has been no direct evidence for involvement of the 2–5A system in controlling cell growth. However, we have recently found that constitutive expression of a 2–5A synthetase cDNA, in addition to providing antiviral activity, also reduces cellular proliferation ( G. Duckworth-Rysiecki, D.R.Gewert and B.R.G. Williams, submitted). Here, we demonstrate that an element in the 5′ flanking region of the 2–5A synthetase gene that confers IFN inducibility is also responsive to PDGF and binds nuclear factors from PDGF-treated cells. These data strongly suggest that activation of the 2–5A synthetase gene plays a central role in the mitogenic response.

## MATERIALS AND METHODS

### Cells, Interferons, and Growth Factors

COS-1 cells were purchased from ATCC. Balb/c-3T3 cells (clone A31) were generously provided by Dr. C.D. Stiles, Dana Farber Cancer Institute, Boston. The cells were grown in modified minimum essential medium supplemented to 10% (v/v) with fetal calf serum. Homogeneous preparations of recombinant human IFN-$\alpha$2B and $\Delta$4 $\alpha$2,$\alpha$1 were provided by Drs. P. Trotta and T. Nagabhushan, Schering Corp. NJ. The recombinant human IFN-$\Delta$4a2,a1 is highly active in mouse cells [8]. Partially purified PDGF was isolated from human platelets as described [9]. Recombinant PDGF was purchased from AmGen Inc. (Thousand Oaks, CA). Sheep polyclonal antisera to mouse IFN-$\alpha/\beta$ was kindly provided by Dr. Ara Hovanessian, Pasteur Institute, Paris.

### Construction of 2–5A Synthetase/Cat Fusion Genes

All manipulation of DNA and RNA was as according to standard methods [10]. The plasmids p(−155/+1) and $p(Alu)_{FF}$ were constructed by inserting the Sau3A fragment (−155/+1) (into the Hind III site of pSVOcat) and the Alu I restriction fragment (−113/−74, see Fig. 1.), into the filled-in Bam HI site of the plasmid pBLcat2 [11].

### Transient Transfection Assays

These were carried out as described [12]. Immediately following transfection, cells were treated with IFN or growth factors for 24 h. Platelet extract was added at a level (1/50 dilution) that stimulated $^3$H-thymidine incorporation in quiescent 3T3 cells by 40- to 50-fold. IFN-$\Delta$4$\alpha$2,$\alpha$l was used at 2,000 IU/ml.

The analysis of CAT activity was exactly as described by Sleigh [13].

### Gel Retardation Assays

Nuclear extracts were prepared according to the methods of Dignam et al. [14] and were used in gel retardation assays as described by Carthew et al. [15].

## RESULTS

The role of 5′-flanking sequences in conferring IFN-responsiveness on the 2–5A synthetase gene was established by fusing restriction fragments derived from the

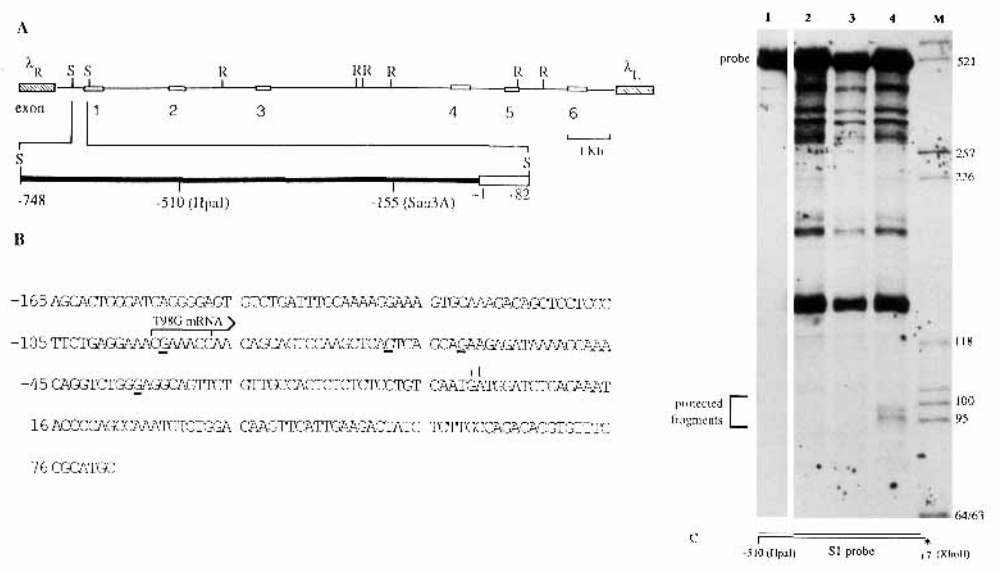


Fig. 1. Structure of the 2-5A synthetase gene and its nucleotide sequence flanking the transcriptional and translational start sites. **A:** Genomic clone (1) used to isolate 5′ flanking sequences. **B:** Nucleotide sequence from −165 to +82. Presumed translational start site is +1. IFN-induced transcriptional start sites determined in **C** are indicated above the sequence. The 5′ termini of reported 2-5A synthetase cDNAs are underlined. C: S-1 nuclease analysis of poly A+ RNA prepared from T98G (human glioblastoma) cells. The probe indicated was annealed with no RNA (**Lane 1**), tRNA (**Lane 2**), RNA from untreated cells (**Lane 3**), or RNA from IFN-treated cells (**Lane 4**) in 80% formamide at 50°C for 16 h. Hybrids were treated with S1, and digestion products were analysed on a sequencing gel. **Lane M** is labeled markers.

5′ 900 nucleotides of λ clone H2-5A,2 (Fig. 1A). S1-nuclease analysis (Fig. 1C) positioned the transcription start sites within this sequence to at least two sites, between positions −88 and −94. The 5′ termini reported for several cDNA clones is indicated (Fig. 1B). Since there is no obvious TATA box in this region and no single transcription initiation site, the sequence numbering uses the presumed initiator ATG for reference (Fig. 1B).

Different restriction fragments were isolated and cloned into the HindIII site of the vector pSVOcat [16], and transient transfection assays were used to determine regions conferring IFN responsiveness. These studies [12] (Fig. 2) determined the region essential for induction to be between −155 and −69. The response to IFN in these assays could be inhibited by cotransfection of this region unlinked to the cat gene suggesting that titratable factors are involved in activation of the 2-5A synthetase promoter. Gel retardation assays [12] confirmed that DNA binding factors specifically bound to this region and defined a 40-base pair AluI subfragment (−113/−74). To determine whether this sequence responded in vivo to IFN, it was linked to a heterologous promoter (HSV tk) situated upstream from the cat structural gene. The constructs were then transfected into CV-1 cells, and CAT activity was assayed after exposure to IFN. The results (Fig. 2) show that this element conferred a significant response to IFN on the tk promoter compared to the −155/+1 fragment in pSVOcat (Fig. 2). Two copies of this element cloned in front of the tk promoter produced about twice the IFN-induced CAT activity as observed for a single copy. The same

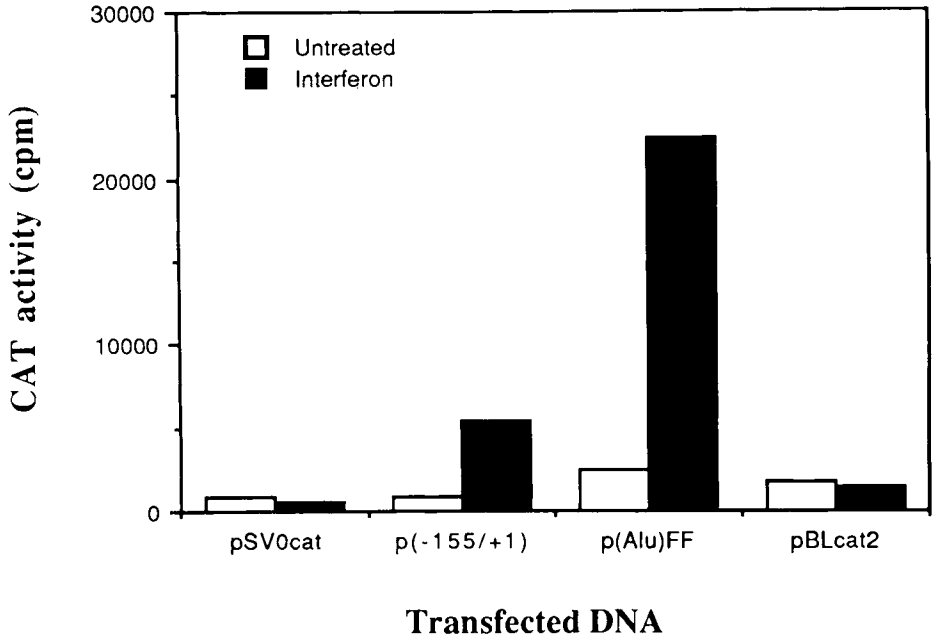


Fig. 2. Upstream elements of the 2-5A synthetase gene confer IFN inducibility. The −155/+1 (Fig. 1) Sau3A restriction fragment was cloned into pSVOcat vector in single forward orientation [12]. The −113/−74 (Fig. 1) AluI restriction fragment was cloned 5′ of the tk promoter in the pBLcat2 vector [11], in double copy forward, $(p(Alu)_{FF})$ orientation. Transient expression of CAT activity in response to IFN-$\alpha_2$ (2,000 units/ml) was assayed in CV-1 cells. Each transfection used 10 $\mu$g of indicator plasmid plus 15 $\mu$g of carrier pUC18 plasmid DNA.

element cloned in the reverse orientation with respect to the tk promoter was also active [12]. Thus, the sequences contained within −113/−74 can confer orientation-independent IFN-inducibility on an heterologous promoter.

The 2-5A synthetase gene may also be induced by PDGF as a primary response [6]. In the well-characterized mitogenic response of Balb/c-3T3 cells to PDGF, 2-5A synthetase mRNA and enzyme levels are elevated early after stimulation. This response is also seen with 40% fetal calf serum (M. Garcia-Blanco and C.D. Stiles, pers. commun.). To determine whether the 2-5A synthetase IFN-response element could also respond to growth factors, we transfected the cat constructs into 3T3 cells and measured CAT activity following treatment with platelet extract (P.E., partially purified PDGF) [9], IFN$\alpha$2$\alpha$l and fetal calf serum. The results (Table I) indicate that there is a significant induction of CAT activity in cells treated with platelet extract compared to untreated cells. This induction is somewhat less than that seen with IFN in these cells (Table I), and was identical with that seen with recombinant PDGF (data not shown). Neither EGF nor TNF stimulated cat expression above background (unpublished observations). The induction of the 2-5A synthetase by PDGF occurs in the presence of protein synthesis inhibitors and with the same kinetics as that observed with IFN [6] (M. Garcia-Blanco and C.D. Stiles, pers. commun.). This suggests but does not prove that the response to PDGF is a primary response to PDGF, rather than a secondary response to endogenous IFN. However, to test this more directly, we added anti-IFN sera to the transfections at the same time as exposure

**TABLE I. Induction of Synthetase/Cat Fusion Genes by IFN and PDGF**

| Inducer | CAT activity (cpm) |
|---|---|
| None | 1501 (174) |
| IFN | 8567 (931) |
| P.E. | 2573 (616) |
| P.E. + Ab | 1677 (211) |
| FCS | 3157 (1098) |

IFN, interferon at 2,000 I.U./ml; P.E., platelet extract; P.E. + Ab, platelet extract plus anti-mouse IFN-$\alpha/\beta$ serum; FCS, fetal calf serum, 40%. Values shown are the means of duplicate transfections, with the variation about each mean bracketed (cpm).

to platelet extract. Surprisingly, this inhibited the PDGF-directed response (Table I). Consequently, we conclude that this response is likely mediated by endogenous IFN.

The ability of the AluI fragment of the 2–5A synthetase gene to be activated by PDGF in mouse cells (Table 1) prompted us to test whether PDGF is able to modulate the binding of nuclear factors in these cells. A gel retardation assay using nuclear factors from IFN-treated or PE-treated cells is shown in Figure 3. IFN clearly modulates the binding of nuclear factors from mouse cells to a synthetic 2–5A synthetase IFN response element (Fig. 3). Platelet extract also modulates factor binding, albeit to a lesser extent. The antibody experiment shown in Table I would suggest that this PDGF effect on the binding of nuclear factors to the 2–5A synthetase gene is occurring through the induction of IFN.

## DISCUSSION

We have identified a 40-base pair region of the 2–5A synthetase promoter, which is required for IFN-activation of this gene in vivo and which binds IFN-modulated nuclear factors in vitro. This element is also responsive to PDGF and binds nuclear factors modulated by PDGF. Since factor binding to this element is specifically competed by a related element from another IFN-induced gene, 6–16 [17] (unpublished observations) we predict that IFN-responsive genes containing similar elements [12] will also be regulated by PDGF.

It has recently been demonstrated that PDGF induces the expression of 2–5A synthetase RNAs in mouse cells with kinetics that are similar to those shown by IFN (M. Garcia-Blanco and C.D. Stiles, pers. commun.). Thus, the 2–5A synthetase pathway is activated early after stimulation of cell growth by PDGF. Since the PDGF response could be inhibited by antibodies to mouse IFN, this activation appears to be mediated through the induction of endogenous IFN by PDGF. This would appear to be in accord with previous data with other mitogens [18] in which 2–5A synthetase induction was demonstrated to be at least partially due to the induction and action of endogenous IFN. However, the induction of 2–5A synthetase by PDGF has previously been observed to be insensitive to both cycloheximide and anisomycin treatments. Since neither of these drugs provide an absolute inhibition of protein synthesis, it is possible that sufficient IFN is produced following exposure to PDGF to mediate synthetase gene induction. To account for the previously observed rapid kinetics of PDGF induction of 2–5A synthetase, a rapid response by both the mouse IFN and 2–5A synthetase genes is required. We have previously shown that the 2–5A synthetase

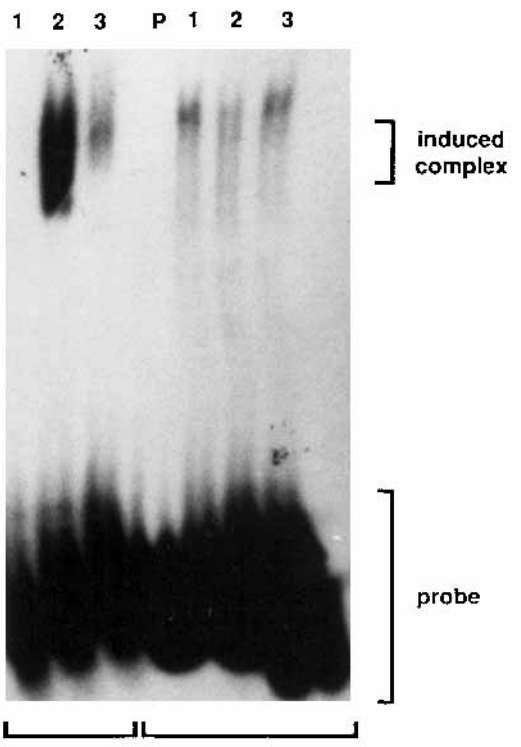


Fig. 3. Band shift mobility assay using nuciear extracts from IFN and PDGF-treated cells; 5 $\mu$g of nuclear extract from untreated cells (**Lane 1**), or cells treated for 30 minutes with IFN (**Lane 2**) or P.E. (**Lane 3**) was incubated with an end-labeled 29-bp oligonucleotide probe spanning a mouse 2-5A synthetase IFN response sequence [19] (**left three lanes**) or with a 20-bp oligonucleotide representing the serum responsive element of the c-fos proto-oncogene [20] (**right four lanes**). **P,** Oligonucleotide probe incubated in the absence of nuclear extract.

gene is induced within 5 min following IFN treatment [5]. While such an immediate response to IFN inducers has not been reported, the rapid PDGF stimulation of c-fos protein accumulation suggests that an early activation of the IFN/2-5A synthetase system is possible.

The 2-5A system has been implicated as an important intracellular effector, mediating between extracellular signals and cellular physiological responses [7]. Direct evidence for such a role has been lacking with the possible exception of the PDGF stimulation of 2-5A synthetase described by Stiles and coworkers [6]. Our data presented here strongly suggest that this induction is mediated by IFN. Nonetheless, the physiological response to PDGF ultimately resulting in mitogenesis also includes activation of the 2-5A system.

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